Lactobacillus rhamnosus STRAIN AND USES THEREOF

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates to a novel lactic acid-forming microorganism which exhibits excellent probiotic properties. The present invention also relates to the uses of the novel microorganism as a probiotic in food, beverage, animal feed and/or dietary supplement compositions, and as a medicament in controlling the colonization of undesirable intestinal microorganisms in the alimentary tract of a mammal.

2. Description of the Related Art

The oral administration of large numbers of *Lactobacillus rhamnosus*, such as *L. (casei subsp.) rhamnosus* GG (ATCC 53103), to a mammal has been found helpful to maintain or even enhance the healthy state of the mammal. It is believed that *L. rhamnosus*, when ingested, would colonize transiently on the intestinal mucosa, which results in inhibition of the growth of pathogenic bacteria and viruses (such as rotavirus), stabilization of gut permeability, and suppression of allergic reactions in food hypersensitivity. The bacterium is particularly effective in alleviating the symptoms of gastroenteric disorders, such as diarrhea, by eliciting nonspecific humoral immune response in hosts.

L. rhamnosus, reported in 1989 as a new species derived

from *L. casei*, shares similar phenotypes with two other members of the *Lactobacillus* genus, *i.e.*, *L. casei* and *L. paracasei*. The three species can be further distinguished in terms of the differences in the genes encoding ribosomal RNAs. Approaches have been conducted based on this finding. For example, Rodtong *et al.* recognized the species-uniqueness of 16S rDNA and developed a ribotyping process to differentiate *Lactobacillus* strains (Rodtong, S. and Tannock, G. W. (1993) *Applied and Environmental Microbiology* **59**: 3480-3484). Taking advantage of the convenience and effectiveness of polymerase chain reaction (PCR), Ward *et al.* and Alander *et al.*, on the other hand, used different sets of primers to identify *L. rhamnosus* based on the sequence polymorphism of 16S rDNA (Ward, L. J. H., and Timmins, M. J. (1999) *Letters in Applied Microbiology* **29**: 90-92; and Alander, M. *et al.*, *Applied and Environmental Microbiology* **65**: 351-354).

According to the present invention, the inventor has identified a novel strain of *L. rhamnosus* (hereinafter referred to as strain Tcell-1) which is phylogenetically distinct from the published strains in the species and exhibits excellent probiotic properties.

SUMMARY OF THE INVENTION

It is a primary object of the present invention to provide a novel strain of *L. rhamnosus*. In the

experiments performed in the invention, the inventor has characterized the phylogenetic distinction of the bacterial strain and demonstrated the desired probiotic properties thereof.

Another object of the present invention is to provide a composition containing the bacterium strain according to the invention and a suitable excipient for the manufacture of foodstuffs, such as beverages, food, animal feed, and dietary supplements.

Still another object of the present invention is to provide a pharmaceutical composition comprising the bacterium strain according to the invention, as well as to provide a method for the treatment or prophylaxis of gastroenteric disorders in a subject by administering such a composition to the subject.

BRIEF DESCRIPTION OF THE DRAWINGS

These and other features and advantages of this invention will become apparent from the following detailed description of this invention, with reference to the accompanying drawings, in which:

Figs. 1A-C demonstrate the enteroscopic sampling from the upper jejunum and rectum tissues of a volunteer;

Fig. 2 is a fermentation profile of the bacterial strain according to the present invention;

Fig. 3A is a restriction map of the chromosomal DNA from the bacterial strain according to this invention;

Fig. 3B shows the result of Southern analysis of Fig. 3A using *E. coli* MRE600 16S+23S rDNA as the probe;

Fig. 4 shows the result of PCR analysis using the primers designed by Ward & Timmins, in which the DNA extracted from the bacterial strain according to the invention (lane 2) and water (lane 3; as a negative control) was subjected to PCR;

Fig. 5 shows the result of PCR analysis using the primers designed by Alander *et al.*, in which two sets of the species-specific primers, rham-rham2 (lanes 2-3) and rham-casei (lanes 4-5) were used in the PCR; and

DETAILED DESCRIPTION OF THE INVENTION

In accordance with the present invention, a strain of *L. rhamnosus* was isolated from the intestinal specimens donated by domestic volunteers. In a preliminary process, the microorganisms from the specimens were screened by a series of selective media, among which MRS agar medium and Rogosa SL agar medium exclusively allow the proliferation of *Lactobacillus*. The bacteria selected according to the above procedure were subjected to a four-step screening strategy for identifying *L. rhamnosus*:

Step 1: fermentation patterning using an API 50CHL kit (BioM'erieux, Lyon, France);

Step 2: ribotyping according to the method described in Rodtong *et al.* (supra), in which the total DNAs

extracted from the microorganisms were treated with restriction enzymes *EcoR* I , *Bcl* I , *Bgl* II or *Hind*III and detected by the rDNA probe of *Escherichia coli* subsequent to Southern blotting, so that the restriction fragment fingerprints of the suspected microorganisms can be obtained and compared with those derived from the *L. rhamnosus* DNA;

Step 3: PCR analysis according to the method described in Ward *et al.* (supra), in which a universal primer Y_2 (5'-CCCAC TGCTG CCTCC CGTAG GAGT-3') and a species-specific primer rham (5'-TGCAT CTTGA TTTAA TTTTG-3') were used in the reaction such that a major product of 290 bp will be produced when the chromosomal DNA of *L. rhamnosus* appears in the reaction mixture; and

Step 4: PCR analysis according to the method described in Alander *et al.* (supra), in which a pair of species-specific primers, rham (as indicated in Step 3) and rham2 (5'-CCGTC AATTC CTTTG AGTTT-3'), will amplify a specific product of 863 bp in the presence of *L. rhamnosus* DNA.

Accordingly, the bacterial strain according to this invention was identified to belong to the species *L. rhamnosus*. However, sequencing of the 863 bp product obtained in the Step 4 leads to a surprising finding that the bacterial strain according to this invention is phylogenetically distinct from all the published strains in the species *L. rhamnosus*.

The bacterial strain thus identified was designated as "*Lactobacillus rhamnosus* Tcell-1" and was deposited in the Culture Collection and Research Center (CCRC) of the Food Industry Research and Development Institute (FIRDI), Hsinchu, Taiwan, R.O.C. under the accession number CCRC 910145 (on April 14, 2000). The bacterium was also deposited at the American Type Culture Center (ATCC) with accession number PTA-2406 on August 22, 2000 under the terms of the Budapest Treaty on the International Recognition of the Deposit of Microorganism for the Purpose of Patent Procedure.

Further studies concerning the probiotic properties of the strain Tcell-1 were also conducted. The results reveal that the bacterial strain according to the present invention can survive and grow well even in the stringent environment that an ingested bacterium would encounter in the gastrointestinal tracts, including extremely acidic pH and a high level of bile. The ability of *L. rhamnosus* Tcell-1 to resist certain antibiotics is apparently beneficial to administration of the bacterium to a subject who is required to take antibiotics. The superiority of the bacterial strain is further reinforced by its capability of inhibiting the growth of various enterobacteria.

In view of the advantageous properties mentioned above, the bacterial strain according to the present invention is suitable for acting as a probiotic.

According to the present invention, the strain Tcell-1 can be formulated into a broad variety of edible materials, including beverages, such as fluid milk, fermented milk, yogurts, fruit juices and sports drinks; food, such as ice cream, cheese and snacks; animal feed; dietary supplements; and infant formulas. Apparently, it is appreciable to those skilled in the art that the bacterial strain of this invention can be formulated in any suitable form by conventional methods for human or non-human animal's uptake. More preferably, the bacterial strain of this invention is formulated into the edible material in combination with other probiotic organisms, such as *L. acidophilus*, *L. brevis*, *L. casei*, *L. plantarum*, *L. salivarius*, *L. bifidus*, *L. bulgaricus*, *L. causasicus*, *Streptococcus lactis* and other strains of *L. rhamnosus*, or a combination thereof. In addition, *L. rhamnosus* Tcell-1 is preferably formulated together with lactosucrose, chitin, chitosan, manitol, yogurt powder or a combination thereof.

L. rhamnosus Tcell-1 can also be used alone or with other active ingredients as a medicament in controlling the colonization of undesirable intestinal microorganisms in the alimentary tract of a mammal, to alleviate the conditions caused thereby. The composition can be formulated in solution, emulsion, powder, tablet, capsule or other adequate forms for oral administration.

The following examples are given for the purpose of illustration only and are not intended to limit the scope of the invention.

THE PREFERRED EMBODIMENTS OF THE INVENTION

Example 1: Isolation of *L. rhamnosus* Tcell-1

Six healthy adults, aged from 25-45 and having no addiction to alcohol or smoking or chronic use of a drug, participated voluntarily in this experiment. None of them are vegetarians nor have abnormal dietary habit. The voluntary donors were subjected to fasting for 12 hours before enteroscopic sampling. Three biopsy specimens, each about 2 mm^2 in size, were picked up from different sites in the upper jejunum and rectum of each donor (Figures 1A-C). The tissue specimens were then washed with physical saline (0.9% NaCl in distilled water) and stored in an ice-cold storage solution (0.9% NaCl, 0.1% Peptone, 0.1% Tween-80 and 0.02% Cysteine) for further analysis. The specimens were treated in an ultrasonic bath for 5 minutes and agitated vigorously for an additional 2 minutes. The obtained suspensions were undiluted or diluted in five- or ten-fold, and aliquots of the preparations were spread on the following solid media to obtain the profiles of enterobacteria contained therein (see also Johansson *et al.*, *Applied and Environmental Microbiology* **59(1)**: 15-20).

1. Brain heart infusion agar (purchased from Difco),

which is an enriched medium for aerobically or anaerobically culturing the entire population of enterobacteria at 37℃ for 3 days;

2. MRS agar (Difco) for anaerobically culturing *Lactobacillus* at 37℃ for 5 days;

3. Phenol ethanol agar (Difco) for aerobically or anaerobically culturing the Gram(+) bacteria at 37℃ for 3 days;

4. Azide blood agar (purchased from Oxoid) for aerobically culturing *Streptococcus* at 37℃ for 2 days;

5. Slanetz-Bartley agar (Oxoid) for anaerobically culturing *Enterococcus* at 37℃ for 2 days;

6. Violet red bile glucose agar (Oxoid) for culturing *Enterobacteriaceae* at 37℃ for 2 days;

7. Rogosa SL agar (Difco) for anaerobically culturing *Lactobacillus* at 37℃ for 5 days; and

8. Reinforced clostridial agar (Difco) for anaerobically culturing *L. bifidus* at 37℃ for 5 days.

The results are shown in Table 1.

Table 1

In the upper jejunum specimens:

Name of the Donor	Medium†														
	BHIA			MRS			PEA			ABA			SBA		
	1x‡	5x	10x	1x	5x	10x	1x	5x	10x	1x	5x	10x	1x	5x	10x
JF	3	0	0	0	0	0	0	0	0	1	0	0	0	0	0
JS	M§	30	1	0	0	0	0	0	0	M	26	0	52	6	3
HK	0	0	0	0	0	0	0	0	0	0	0	6	0	0	0
V	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
B	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
RG	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Name of the Donor	Medium								
	VRBGA			RA			RCA		
	1x	5x	10x	1x	5x	10x	1x	5x	10x
JF	0	0	0	0	0	0	M	0	0
JS	0	0	0	0	0	0	M	0	21
HK	0	0	0	0	0	0	0	0	0
V	0	0	0	0	0	0	0	0	0
B	0	0	0	0	0	0	0	0	0
RG	0	0	0	0	0	0	0	0	0

In the rectum specimens:

Name of the Donor	Medium														
	BHIA			MRS			PEA			ABA			SBA		
	1x	5x	10x	1x	5x	10x	1x	5x	10x	1x	5x	10x	1x	5x	10x
JF	M	47	6	M	23	1	0	9	0	M	1	0	28	0	0
JS	M	15	1	M	8	0	0	12	0	M	0	1	0	1	0
HK	M	1	0	0	0	0	0	0	0	12	0	0	0	0	0
V	M	0	0	0	0	0	0	0	0	30	0	0	0	0	0
B	M	28	11	M	67	3	0	10	0	M	14	0	M	4	4
RG	M	0	0	0		0	0	0	0	1	0	0	0	0	0

Name of the Donor	Medium								
	VRBGA			RA			RCA		
	1x	5x	10x	1x	5x	10x	1x	5x	10x
JF	M	M	M	0	0	0	M	M	M
JS	M	15	0	0	0	0	M	M	0
HK	0	0	0	0	0	0	M	0	0
V	0	0	0	0	0	0	M	0	0
B	M	M	M	M	0	0	M	M	M
RG	0	0	0	0	0	0	M	0	0

† The abbreviation BHIA represents brain heart agar; MRS represents MRS agar; PEA represents phenol ethanol agar; ABA represents azide blood agar; SBA represents Slanetz-Bartley agar; VRBGA represents violet red bile glucose agar; RA represents Rogosa SL agar; and RCA represents reinforced clostridial agar.

‡ 1X, 5X and 10X are the dilution folds of the bacterial suspensions.

§ The letter M indicates that the number of bacterial colonies on the medium plate is higher than 100.

As shown in Table 1, the profiles of enterobacteria in the upper jejunum and rectum specimens are quite different.

Example 2: Isolation of *Lactobacillus*

From the MRS and Rogosa SL agar media in Example 1, 200 colonies were picked up randomly and transferred separately to fresh MRS agar media containing 1% $CaCO_3$. After incubation, the colonies surrounded by clear zones were picked up, and each of them was transferred to a basal MRS agar medium supplemented with 1% rhamnose and 0.05% chlorophenol red. Finally, the yellowish colonies, presumably constituted by *Lactobacillus*, were picked up

and further transferred to fresh MRS broth and incubated anaerobically at 37℃ for 2 days for further analysis.

Example 3: Identification of *L. rhamnosus* Tcell-1 as a new strain

(a) Fermentation pattern

The *Lactobacillus* broth prepared in Example 2 was precipitated, washed with distilled water and resuspended in a defined amount of distilled water. The bacterial suspensions thus obtained were investigated using an API 50CHL kit according to the protocol provided by the manufacturer. Upon this procedure, a strain of *L. rhamnosus* was identified based on the fermentation pattern specific to the species (Figure 2) and designated as *L. rhamnosus* Tcell-1.

Total DNA of the strain Tcell-1 was prepared from a 3 ml culture growing in the mid-log phase according to a conventional method described by Sambrook *et al.* (Sambrook, J. *et al.*, *Molecular Cloning, A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, 1989), and resuspended in 50 μl of TE buffer (1 mM EDTA, 10 mM Tris-HCl, pH 8.0). The DNA solution thus obtained was used in the following analyses for further investigation of the strain Tcell-1.

(b) Ribotyping analysis

10 μl aliquots of the DNA were digested by restriction endonucleases, *EcoR* I, *Bcl* I, *Bgl* II and *Hind* III, respectively,

for 3 hours. The digested products were loaded into the wells of a 0.8% agarose gel, and electrophoresis was carried out at 5 V/cm for 2 hours. The gel was then stained with ethidium bromide, and an image of the gel was obtained as shown in Figure 3A. The DNAs on the gel were denatured and transferred to a nylon-based membrane (Hybond-N$^+$, Amersham) as described by Sambrook *et al.* (supra). To prepare the probe for Southern analysis, 1 μl of *E. coli* MRE600 16S+23S rRNA (purchased from Boehringer Mannheim) was used as the template which was amplified via incorporation of [α-^{32}P] dCTP by AMV reverse transcriptase (Bethesda Research Laboratories) and random primers. Blots were hybridized at 68℃ for 16-24 hours in a hybridization solution containing 5X SSC, 1X Denhardt's solution, 1% SDS and 100 mg/ml of Harpin sperm DNA, washed properly to enhance the signal-to-noise ratio, and subjected to autoradiography. As shown in Fig. 3B, the ribotype of the strain Tcell-1 was in perfect agreement with the typical pattern of *L. rhamnosus* as described by Rodtong *et al.* (supra).

(c) PCR analysis using the Ward & Timmins' primers

To an 1 ml eppendorf, 1 μl of Tcell-1 DNA harvested in Example 3(a), 1 μl of primer Y_2, 1 μl of primer rham, 0.5 μl of DynaZyme II (Finnzymes Oy) and each dNTP (dATP, dTTP, dCTP and dGTP) at 100 μM were added. The reaction mixture was added with distilled water to a final volume of 50 μl and further overlaid with mineral oil. The

reaction mixture was placed in a GeneAmp® PCR System 2400 thermocycler (Perkin Elmer) and thermocycled under the following conditions:

Initial condition:	94℃ for 3 min.
	45℃ for 45 sec.
	72℃ for 1 min.
Thermocycling:	94℃ for 45 sec.
	45℃ for 45 sec.
	72℃ for 1 min.
Number of thermocycles:	30
Chain extension:	94℃ for 45 sec.
	45℃ for 45 sec.
	72℃ for 5 min.

Following thermocycling, the amplified products were separated on a 0.2% agarose gel. The gel was stained with ethidium bromide, and a major amplicon of 290 bp was observed on the gel under a UV light source (Fig.4)

(d) PCR analysis using the Alander's primers

The PCR in Example 3(c) was repeated except that the Ward & Timmins' primers were replaced with the rham and rham2 primers designed by Alander *et al.* (supra). The electrophoresis analysis on a 0.2% agarose gel revealed that the amplified products contain a major band of 863 bp (Fig. 5), which, as described above, was reported to be a critical indicator to identify *L. rhamnosus*.

The experiments conducted in Examples 3(a)-(d) conclude that the biochemical and genetic traits of the

strain Tcell-1 matched with those considered belonging to species *L. rhamnosus*.

(e) Differentiation of strain Tcell-1 from other *L. rhamnosus* strains

Using TOPO TA cloning™ kit (Invitrogen), the 863 bp product obtained in Example 3(d) was cloned into a pCR-TOPO™ vector according to the protocol provided by the manufacturer. The resultant plasmid was then introduced into TOP10 One Shot™ electrocompetent cells (Invitrogen) by electroporation. Following proliferation of the transformants in a selective medium, the plasmid was harvested and the 863 bp insert was sequenced.

The sequence was used as a query sequence and searched against a nucleotide sequence database in the GenBank (http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?CMD=search&DB=nucleotide). The sequence alignment shown in Table 2 suggests that the strain Tcell-1 is phylogenetically distinct from all the six *L. rhamnosus* strains available in the GenBank based on the 16S rDNA sequences. The Tcell-1 DNA sequence shown in Table 2, which is 776 bp in size, was designated as SEQ ID No.1.

Table 2

```
symbol comparison table :    genetiq.dat; gap penalty : 4
                1        10        20        30        40        50
TCELL1     TATACACTGGTACCTCCCTAAGTGGGATACATTGAAACAATCTATCCGCATAATCAAGA
                      *** ***           *  *     * *    * *  *  *
AF21761       TTGTACACACCGCCC.GTCACACCATGAGAGTTTGTAACA...CCCGAAGCCGGTG
              *************** ************************   ************
E08782        CTTGTACACACCGCCC.GTCACACCATGAGAGTTTGTAACA...CCCGAAGCCGGTG
               *   *   * *   * *  *  *     **       ***   * **** *
AF18273    CCTTTCTAAGGAAACAGACTGAAAGTCTGACGGAAACCTGCACA...CACGAAACTTTGT
                                 *  * *** ****     **
A61362                      CTAAGGAAACAGACTGAAAGTCTGACG.............
                            ***************************
U32966                      CTAAGGAAACAGACTGAAAGTCTGACG.............
                              ************************
AF12120                       AAGGAAACAGACTGAAAGTCTGACG.............
                              1        10        20
consens                    A C  CC A GA ACAGAC GAAA TCT AC    C C  A

                1        10        20        30        40        50        60
                          70        80        90       100       110
TCELL1     CCGCATGTCTTGCTAAGATGCGTAACTATCGCTTTGGATGACCCCGCGTATAGCTAGTTG
            ** *       *     *  * * * *               **  *  *
AF21761    GCGTAA......CCCTTTTAGGGAGCGA.............GCCGTCTAAGGTGGGACAA
           ******      ** ***  **                     *     * *  **   **
E08782     GCGTAA......CCTTTTAGGGAGCGAG.............CCGTCTAAGGTGGGACAAA
                         **       *                          *
AF18273    TTAGTTTTGAGGGGATTACCCTCAAGCACCC.........TAGCGGGTGCGACTTTGTTC
A61362     ..........................................................
U32966     ..........................................................
AF12120    ..........................................................
consens
```

Table 2 (continued)

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                    70         80         90        100        110        120
                   130        140        150        160        170
TCELL1   TAAGTAACGCTCACCAAGCAATGATGCTAGCCAACTAAGTTGATCGCCACATTGGACTAA
          * * * * *    * **  * *    *  * *       ** *    *   **
AF21761  ATGATTAGGGTGAAGTCGTAACAAGGTAGCCGTAGGAGAACCTGCGGCTGGATCACCTCC
          *  **   *      ************************************
E08782   TGATTAGGGTGAAGTCGT.AACAAGGTAGCCGTAGGAGAACCTGCGGCTGGATCACCTCC
*         *    ** *                *    *   *   **     *     *   *
AF18273  TTTGAAAACTGGATATCATTGTTGTAAATGTTTTAAATTGCCGAGAACACAGGCTATTTG

A61362   ............................................................

U32966   ............................................................

AF12120  ............................................................

consens             A                      A         C         T


                  130        140        150        160        170        180
                   190        200        210        220        230
TCELL1   ACACGGCC.CAAACTCTACGGAGGCAGCAGTAGGAATCTTCCACAATGGACGCAAGTCTG
          *      ****   ** **                  *  **        *  *
AF21761  TTTCTAAG.GAAACAG.ACTGAAAGTCTGA.....CGGAAACCTGCACACACGA
         ******** ******* ************        ******************
E08782   TTTCTAAG.GAAACAG.ACTGAAAGTCTGA.....CGGAAACCTGCACACACGA
         * *       ** *  *   *    *         ** * * * **  ** * *
AF18273  TATGAGTTTCTAATAATAGAAATTCGCAT.....CGCATAACCGCTGACGCAA
                                             * * * **  ** * *
A61362   ..........................................GAAACCTGCACACACGA
                                                 ****************
U32966   ..........................................GAAACCTGCACACACGA
                                                 ****************
AF12120  ..........................................GAAACCTGCACACACGA
                                                    30        40
consens             AA    A   A                 C GAAACCTGCACACACGA
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Table 2 (continued)

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                    190        200        210        220        230        240
                      250        260        270        280        290
TCELL1    ATGGAGCAACGCCGCGTGACTGAAGAAGGCTTTCGGGGCGTAAAACTCTGTTGTTGGAGA
          *                  **  *          *        *          **
AF21761   AACTTTGTTTAGTTTTGAGGGGATTACCCTCAAGCACCCTAGCGGGTG.......CGACT
          ********************** *************** ******       *****
E08782    AACTTTGTTTAGTTTTGAGGGGATCACCCTCAAGCACCCTAACGGGTG.......CGACT
            *  *       *          *   * * *             **          ***
AF18273   GTCAGTACAGGTTAAGTTACAAAGGGCGCACGGTGGATGCCTTGGCACTAGGAGCCGATG
            *  *       *          *   * * *             **
A61362    AACTTTGTTTAGTTTTGAGGGGATTACCCTCAAGCACCCTAGCGGGTG............
          ********************************************
U32966    AACTTTGTTTAGTTTTGAGGGGATTACCCTCAAGCACCCTAGCGGGTG............
          ********************************************
AF12120   AACTTTGTTTAGTTTTGAGGGGATTACCCTCAAGCACCCTAGCGGGTG............
                50         60         70         80         90
consens   AACTTTGTTTAGTTTTGAGGGGATTACCCTCAAGCACCCTAGCGGGTG        GA


                  250        260        270        280        290        300
                    310        320        330        340        350
TCELL1    AGAATGGTCGGCAGAGTAACTGTTGTCGGCGTGACGGTATCCAACCAGAAAGCCACGGCT
                    *****                 **       *      * *   *
AF21761   TTGT.....TCTTTGAAAACTGGATATCATTGTTGTAAATGTTTTAAATTGCCGAGAACA
          ****     ************************  *** ********************
E08782    TTGT.....TCTTTGAAAACTGGATATCATTGTATTAATTGTTTTAAATTGCCGAGAACA
            *       **   *    *     **   *   **   **       *   *  **
AF18273   AAGGACGGAACTAATACCGATATGCTTCGGGGAGCTATAAGTAAGCTTTGATCCGGAGAT
                                              ** *             *    *
A61362    ..............................CGACTTTGTTCTTTGAAAACTGGATATCA
                                        ****************************
U32966    ..............................CGACTTTGTTCTTTGAAAACTGGATATCA
                                        ****************************
AF12120   ..............................CGACTTTGTTCTTTGAAAACTGGATATCA
                                                  100        110
consens                      T           GACT T T CTTT AAAA TCGA A CA
```

Table 2 (continued)

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                    310        320        330        340        350        360
                      370        380        390        400        410
TCELL1    AACTCAGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGATTTATTGGG
           *     *               * *
AF21761   CAGCGTATTTGTATGAGTTTCTAATAA.....................................TA
          *****   **         **      **                                    *
E08782    CAGCG.TATTTGTATGAGTTTCTGAAA.....................................AA
             **      * *       *    *
AF18273   TTCCGAATGGGGGAACCCAGTACACATCAGTGTATT.............................GC
          **
A61362    TTGTTGTAA.......................................................AT
          *********                                                       **
U32966    TTGTTGTAA.......................................................AT
          *********                                                       **
AF12120   TTGTTGTAA.......................................................AT
                                                                          130
consens   TTGT  TA                                                         A

                    370        380        390        400        410        420
                      430        440        450        460        470
TCELL1    CGTAAAGCGAGCGCAGGCGGTTTTTTAACTCTGATGTGAAAGCCCTCGGCTTAACCGAGG
             *     * ****                          ** * **      *  *
AF21761   GAAATTCGCATCGCA.....................TAACCGCTGACGCAAGTC....
          ***************                     ******************
E08782    GAAATTCGCATCGCA.....................TAACCGCTGACGCAAGTC....
                  *   **                      ** *   *
AF18273   CTGCAAGTGAATACA.....................TAGCTTGTTGGCGGCAGACGCG
           *   *        *
A61362    GTTTTAAATTGCCGA.....................................................
          ***************
U32966    GTTTTAAATTGCCGA.....................................................
          ***************
AF12120   GTTTTAAATTGCCGA.....................................................
                  140
consens   GTT TA   AGC CA                      A     T
```

Table 2 (continued)

```
                    430        440        450        460        470        480
                      490        500        510        520        530
TCELL1    AAGTGCATCGGAAACTGGGAAACTTGAGTACAGAAGAGGACAGTGGAACTCCATGTGTAG
          *    **    *        *       *     ** *           *    *
AF21761   AGTACCAGGTAAGTTACAAAGGGCGCACGGTGGATGCCTTGGCACTAGGAGC.......C
          *****  * ****************************************        *
E08782    AGTACAGGTTAAGTTACAAAGGGCGCACGGTGGATGCCTTGGCACTAGGAGC.......C
           * *   *  *  *  **     **** *  *          * * *    *        *
AF18273   GGGAACTGAAACATCTCAGTACCCGCAGGAAGAGAAAGAAAACTCGATTCCCATAGTAGC
            ****  *      *              ** *  *  **  * *
A61362    ..GAACACAGCGTATTTGTATGAGTTTCTAATAATAGAAATTCGCATC...........
            ******************************************
U32966    ..GAACACAGCGTATTTGTATGAGTTTCTAATAATAGAAATTCGCATC...........
            ******************************************
AF12120   ..GAACACAGCGTATTTGTATGAGTTTCTAATAATAGAAATTCGCATC...........
             150        160        170        180        190
consens     GAACA AG    ATT G A G   T ACTAA AATA   AA  C C A     C

                    490        500        510        520        530        540
                      550        560        570        580        590
TCELL1    CGGTGAAATGCGTAGATATATGGAAGAACACCAGTGGCGAAGGCGGCTGTCTGGTCTGTA
               *      * *    ***  **     *  * * *    *                  *
AF21761   GATGAAGGACGGAACTAATACCGATATGCTTCGGGGAGCTATA.................A
          ****************************************                    *
E08782    GATGAAGGACGGAACTAATACCGATATGCTTCGGGGAGCTATA.................A
          *  **  ** *     *   ** * *       *   ***
AF18273   GGCGAGCGAAGTGGGAAGAGCCCAAACCGAGAAGCTTGCTTCTCGGGGTTGTAGGACTGG
                       * *  * * *  **  *      * *
A61362    .............GCATAACCGCTGACGCAAGTCAGTACAGG
                       ****************************
U32966    .............GCATAACCGCTGACGCAAGTCAGTACAGG
                       *************************
AF12120   .............GCATAACCGCTGACGCAAGTCAGTACA
                             200        210      218
consens                GCATAA CGCA ACGCA   G GT CA
```

Table 2 (continued)

```
                    550        560        570        580        590        600
                      610        620        630        640        650
TCELL1    ACTGACGCTGAGGCTCGAAAGCATGGGTAGCGAACAGGATTAGATACCCTGGTAGTCCAT
               *  ***  *    *                *       *  **** *      **
AF21761   GTAAGCTTTGATCCGGAGATT...........TCCGAATGGGGGAACCCAGTA....CAC
          ********************             ********************    ***
E08782    GTAAGCTTTGATCCGGAGATT...........TCCGAATGGGGGAACCCAGTA....CAC
            *          **  ** *             * * * *  *    * * *      *
AF18273   ACATTGGAGTTACCAAAGTTCG...........ACGTAGTCGAAGTCAGCTGGAAAGCTGC

A61362

U32966

AF12120

consens             C                                    C G

                    610        620        630        640        650        660
                      670        680        690        700        710
TCELL1    GCCGTAAACGATGAATGCTAGGTGTTGGAGGGTTTCCGCCCTTCAGTGCCGCACTAACGC
            *          * ***  *    ** *    *  *                  *  ****
AF21761   ATCAGTG.....TATTGCCTGCAAGTGAATACATAGCTTGT......TGGCGGCAGACGC
          *******        *  *    **   *   *    *                * *    *
E08782    ATCAGTGTGTTGCTTGTCAGTGAATACATAGCTGGCCGGCG......GCCAGACGCGGGG
            **  *          *  ** * **  * * *                  * *
AF18273   GCCATAGAAGGTGAAAGCCCTGTAAACGAAACGGCGGACTC....TCCGTCCAGGATCCT

A61362

U32966

AF12120

consens   C              C
```

Table 2 (continued)

```
                    670        680        690        700        710        720
                      730        740        750        760        770    776
TCELL1    ATTAAGCATTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGG
           * *                          **   *  **
AF21761   GGGGAACT.....................GAAACATCTAAG
             *                         *   *  **
E08782    AACTGAAA.....................CATCTAAGTACCCGGA
           * *                         *     * *     *
AF18273   GAGTACGGCGGAACACGTGAAATTCCGTCGGAATCCGGGAGGACCATCT

A61362

U32966

AF12120

consens                               A       A
          ~        730        740        750        760        770    778
```

Example 4: Characterization of *L. rhamnosus* Tcell-1

(a) Tolerance of acid

MRS liquid media were prepared at pH 2, 3, 4, 5 and 6, respectively, and supplemented with 0.3% bile salt. To 1.5 ml of each medium, 10^6 Tcell-1 cells were inoculated and incubated anaerobically at 37℃. Samples were collected at 0 and 4 hours after the inoculation, and the populations of the microorganisms in each culture were assessed with reference to the optical density at 620 nm. The results are shown in Table 3.

Table 3

Incubation time	OD_{620}				
	pH2	pH3	pH4	pH5	pH6
0 hour	0.01	0.018	0.010	0.014	0.012
4 hour	0.01	0.018	0.016	0.025	0.030

As shown in Table 3, the growth rate of *L. rhamnosus* Tcell-1 remarkably reduced as the cultures were acidified to a pH at which the ingested substances would encounter in an animal stomach, *i.e.*, a pH of below 3. Surprisingly, the cells incubated in such an acidic environment for 4 hours can still restore their normal growth if transferred to a fresh MRS medium at pH 6.0 (data not shown). The data indicate that *L. rhamnosus* Tcell-1 can tolerate the attack of gastric acid.

(b) Tolerance of bile salt

Example 4(b) was repeated except that the MRS liquid

media contained bile salts at concentrations of 0.1, 0.2, 0.3 and 0.4%, respectively, while the pH of the media was constantly set at 2.5. The results are shown in Table 4.

Table 4

Incubation time	OD_{620}			
	0.1%	0.2%	0.3%	0.4%
0 hour	0.011	0.010	0.013	0.018
4 hour	0.028	0.025	0.023	0.032

From Table 4, it is demonstrated that the growth of *L. rhamnosus* Tcell-1 was sustained at a high level of bile.

(c) Resistance to antibiotics

2-3 ml aliquots of a bacterial suspension from an overnight culture of *L. rhamnosus* Tcell-1 were spread on MRS agar media added with 10 μg/ml of kanamycin, vancomycin, chloramphenicol or ampicillin. After incubation, *L. rhamnosus* Tcell-1, while its growth was attenuated in the media containing chloramphenicol or ampicillin, was found to be tolerant of kanamycin and vancomycin.

(d) Inhibition of the colonization of other bacteria

2-3 ml aliquots of a bacterial suspension from an overnight culture of *L. rhamnosus* Tcell-1 were spread on MRS agar media. Each of the plates was incubated at 30℃ for 22 hours, on which 7 ml of soft agar mixed with 100μl suspension from one of the nine enterobacterial strains listed in Table 5 (purchased from the FIRDI) was

poured.

Table 5

Bacterium	Medium
Enterobacter aerogenes	DIFCO 0001
Clostridium perfringens	brain heart infusion (anaerobically cultured)
Klebsiella pneumoniae	DIFCO 0001
Yersinia enterocolitica	brain heart infusion
Listeria monocytogenes	brain heart infusion
Streptococcus mutans	brain heart infusion
Citrobacter freundii	DIFCO 0001
Shigella dysenteriae	DIFCO 0001
Yersinia ruckeri	DIFCO 0001

The obtained cultures were incubated for an additional 48 hours at 37℃ and observed with bare eyes. Based on the presence of inhibition rings around the colonies of *L. rhamnosus* Tcell-1, the inventor found that the strain Tcell-1 can significantly suppress the growth of *E. aerogenes*, *C. perfringens*, *L. monocytogenes*, *S. mutans* and *C. freundii*. The results strongly suggest that *L. rhamnosus* Tcell-1 exhibits promising probiotic properties for controlling or inhibiting the colonization of the undesired bacteria in the bowel.

Example 5: PROBIOTIC FORMULATIONS CONTAINING *L. rhamnosus* Tcell-1

L. rhamnosus Tcell-1 can be utilized in various forms of foodstuffs, two examples of which are described as follows:

Formula 1:

Ten strains of lactic acid-forming bacteria:

L. acidophilus, L. brevis, L. casei, L. plantarum, L. salivarius, L. bifidus, L. bulgaricus, L. causasicus, Streptococcus lactis and *L. rhamnosus* Tcell-1;

Excipients:

lactosucrose oligo, manitol, chitin & chitosan, yogurt powder;

Natural condensates:

alfalfa, barley and wheat grass juice powder, pure soya lecithin, carrot juice powder, phosphatidyl choline, Hawaiian *Spirulina pacifica*, apple pectin powder, phosphatidyl inositol CGF chlorella, non-dairy probiotic culture: *rhamnosus, acidophilus* in a base of FOS, peace river bee pollen powder, stevia, freeze-dried mango, black currant, dandelion root extract 4:1, beetroot extract, Siberian ginseng extract 0.4%, pacific kelp 4:1 extract, artichoke 4:1 extract 2%, soya extract, bilberry extract 5:1, pineapple extract, cranberry juice extract 18:1, rosehip extract 4:1, lycopene, Milk Thistle Phytosome™, Ginkgo Biloba Phytosome™ and Grape Seed Phytosome™;

Other ingredients:

Vitamin C, calcium, magnesium, zinc.

Formula 2:

Nine strains of lactic acid-forming bacteria:

L. acidophilus, L. brevis, L. casei, L. plantarum,

L. salivarius, *L. bifidus*, *L. bulgaricus*, *L. causasicus* and *L. rhamnosus* Tcell-1;

Other ingredients:

calcium lactate, lactosucrose oligo, lactose, dextrose, powered milk, vegetable oil and small amounts of an emulsifier and natural seasonings

The formulation is coated on peanut and raisin granules to make up a probiotic healthy dessert.

With this invention thus explained, it is apparent that numerous modifications and variations can be made without departing from the scope and spirit of this invention. It is therefore intended that this invention be limited only as indicated by the appended claims.